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Intraepithelial paracrine Hedgehog signaling induces the expansion of ciliated cells that express diverse progenitor cell markers in the basal epithelium of the mouse mammary gland

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A B S T R A C T

The Hedgehog signaling pathway regulates embryo patterning and progenitor cell homeostasis in adult tissues, including epidermal appendages. A role for the Hh pathway in mammary biology and breast cancer has also been suggested. The aim of this study was to analyze Hh signaling in the mouse mammary gland through the generation of transgenic mice that express Sonic Hedgehog (Shh) under the control of the mammary specific WAP promoter (WAP Shh mice). To identify mammary cells capable of activating the Hh pathway we bred WAP Shh mice to Ptch1 lacZ knock in mice, in which the expression of a nuclear targeted β galactosidase reporter protein (β gal) is driven by the endogenous Patched 1 gene regulatory region. After two cycles of induction of transgenic Shh expression, we detected areas of X gal reactivity. Immunohistochemical analysis showed nuclear β gal staining in clusters of mammary cells in WAP Shh/Ptch1 lacZ bitransgenic mice. These were epithelial cells present in a basal location of displastic ducts and alveoli, adjacent to Shh expressing luminal cells, and overexpressed epithelial basal markers keratin 5, 14 and 17 and transcription factor p63. Absence of smooth muscle actin expression and a cuboidal morphology differentiated Hh responding cells from flat shaped mature myoepithelial cells. Groups of cells expressing stem cell markers integrin β3 and keratins 6 and 15 were also detected within Hh responding areas. In addition, we found that Hh responding cells in the mammary glands of WAP Shh/Ptch1 lacZ mice were ciliated and exhibited a low proliferation rate. Our data show the paracrine nature of hedgehog signaling in the epithelial compartment of the mouse mammary gland, where a subset of basal cells that express mammary progenitor cell markers and exhibit primary cilia is expanded in response to secretory epithelium derived Shh.

Introduction

The Hedgehog (Hh) signal transduction pathway is a fundamental regulator of embryonic development and tissue homeostasis. Hh ligands bind to Patched membrane receptors, activating the pathway through release of Smoothened inhibition and subsequent activation of Gli transcription factors, which in turn stimulate expression of downstream target genes. In vertebrates, there are three Hh secreted ligands: Sonic (Shh), Indian (Ihh) and Desert (Dhh) hedgehog, and three Gli proteins. Gli1 works exclusively as an activator, but Gli2 and Gli3 can function as activators or proteolytically processed repressors (Pan et al., 2006; Wang et al., 2000). However, a number of studies show that Gli3 works predominantly as a transcriptional repressor (Wang et al., 2000) while Gli2 works as an activator and is required for the initiation of Hh signaling (Bai et al., 2002). Target genes of Hh signaling include Gli1 and Patched 1 (Ptch1) genes, and their expression has been widely used to assess Hh pathway activity (Jiang and Hui, 2008).

Hedgehog ligands are secreted proteins that can diffuse through several cellular diameters (Ingham and McMahon, 2001). In several different tissues, Hh signaling involves Hh producing cells, that express and secrete Hh, and Hh responding

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cells that express Patched receptors, pointing to paracrine interactions as an important mode of action for this pathway. During prostate development, Shh secreted by epithelial cells in the urogenital sinus activates the Hh pathway on adjacent mesenchymal cells, which in turn elicit paracrine effects on epithelial proliferation and differentiation (Yu et al., 2009) and Hh proteins secreted by the endodermal epithelium during gastrointestinal tract development act as paracrine mitogens to promote the expansion of adjacent mesenchyme (Mao et al., 2010).

The critical role of hedgehog signaling in the development and tissue homeostasis of some epidermal appendages, hair follicle and teeth, is well established (Ingham and McMahon, 2001). Several studies have sought to analyze its implication in the development of the mammary gland, an epidermal appendage that originates during embryonic life but mainly develops during puberty and acquires a functional differentiation with the onset of lactation (Hennighausen and Robinson, 2001). Hh pathway components, including all three Hh ligands (Gallego et al., 2002; Kouros Mehr and Werb, 2006; Lewis et al., 2001; Michno et al., 2003; Velanovich et al., 1999) and downstream transcription factors Gli2 and Gli3, are expressed in the mammary tissue (Hatsell and Cowin, 2006; Lewis et al., 2001). Expression of Hh transcriptional target genes Gli1 and Ptch1, indicative of pathway activation, has also been described in the mammary epithelium and stroma (Gallego et al., 2002; McDermott et al., 2010; Velanovich et al., 1999) but their expression levels are low and expression analysis using lacZ based transcriptional reporter alleles for both Gli1 and Ptch1 suggested that Hedgehog signaling might be absent or repressed in mammary cells throughout development (Hatsell and Cowin, 2006). In addition, we and others found that Shh and Ihh null embryos display normal mammary buds that develop normally when transplanted into cleared fat pads (Gallego et al., 2002; Michno et al., 2003) although functional redundancy between Hh ligands could account for the lack of mammary phenotype in these mutants. Mice lacking functional Gli3 repressor protein show disruption of embryonic mammary glands formation, supporting the notion that Hh signaling repression is necessary during early mammary gland morphogenesis (Hatsell and Frost, 2007). Patch1 gene haploinsufficiency leads to limited Hh pathway activation resulting in mammary ductal dysplasias and hyperplasias (Moraes et al., 2009; Velanovich et al., 1999). In addition, forced ligand independent activation of the Hh pathway in the mammary gland of transgenic mice through the expression of an activated form of Smoothened also results in ductal hyperplasias and dysplasias (Moraes et al., 2007; Visbal et al., 2011) and overexpression of Gli1 in the mammary epithelium of transgenic mice caused impaired lactation and tumor development (Fiaschi et al., 2009; Fiaschi et al., 2007). Collectively, these data suggest that, although Hh pathway repression might be the physiological state in at least some stages of mammary development, Hh pathway misactivation in mammary tissue may occur, leading to hyperproliferation of the epithelial compartment and disturbances of the mammary structure that are reminiscent of lesions found in human breast pathologies (Fiaschi et al., 2009; Velanovich et al., 1999).

Inappropriate Hh signaling has been demonstrated in a variety of tumors (Berman et al., 2003; Karhadkar et al., 2004; Thayer et al., 2003; Theunissen and de Sauvage, 2009; Watkins et al., 2003) and remodeling of the tumoral stroma by tumor epithelium derived Hh ligands acting on mesenchymal cells, that in turn provide a more favorable environment for tumor growth, has been proposed as a commonly occurring cancer promoting mechanism in a number of epithelial tumors (Yauch et al., 2008), including human breast cancer (O'Toole et al., 2011). A mitogenic role for Hh in mammary stem cell self renewal and in elaboration of mammary progenitors has also been described (Li et al., 2008; Liu et al., 2006), suggesting the existence of an intraepithelial Hh signaling mechanism in the mammary tissue, whereby epithelium derived Hh ligands might act directly on mammary epithelial progenitor cells. Unraveling the cellular interactions involved in Hh signaling in the mammary gland will contribute to the characterization of this pathway in mammary tumoral pathology.

Recent work reveals that, in vertebrates, Hh signaling is highly related to the presence of the primary cilium, a microtubule containing subcellular organelle that protrudes from the cell surface (Zhang et al., 2009). Functional genetic screens showed that mutations affecting primary cilia integrity or function in mice produced patterning defects that resembled Hh related phenotypes (Huangfu et al., 2003). Furthermore, Hh pathway components are enriched in the cilia, and cilia are necessary for both activating and repressing functions of Gli proteins (Haycraft et al., 2005; Huangfu and Anderson, 2005). A role for primary cilia has recently been described in mammary branching morphogenesis. Mice bearing a ciliary dysfunction presented decreased ductal extension and diminished secondary and tertiary branching, along with reduced lobular alveolar development during pregnancy and lactation (McDermott et al., 2010). During mammary development, primary cilia are found on luminal epithelial, myoepithelial, and stromal cells in early branching morphogenesis but disappear from luminal epithelial cells in the mature mammary gland (McDermott et al., 2010). It remains to be studied whether this distribution reflects the location of Hh responsive cells in this organ.

We have sought to analyze Hh pathway signaling in the mammary epithelium, trying to determine which cells are responsive to Hh ligands, and what are the effects on the mammary tissue when the pathway is activated in the mouse mammary gland employing a ligand driven Hh pathway activation transgenic model.

Materials and methods

Generation of WAP Shh transgenic mice

The mouse Shh cDNA (a gift from Dr. Phil Beachy, Stanford University) was cloned in an expression vector containing a 1.6 kb fragment of the mouse *WAP* (whey acidic protein) gene promoter sequence, the second intron of rabbit *β globin* gene and rabbit *β globin* gene and SV40 polyadenylation sequences (Gallego et al., 2003). Transgenic mice were generated by microinjection of this construct into C57Bl/6JxDBA/2J F2 embryos using standard techniques. Transgenic founders were identified by Southern blot analysis of tail genomic DNA using mouse Shh cDNA as a probe (not shown). Transgenic offspring were subsequently identified by PCR using primers ShhTg F: 5′ CGT GCT GGT TAT TGT GCT GTC 3′ and ShhTg R: 5′ CCA CTG GTT CAT CAC AGA GAT G 3′ amplifying a fragment of 400 pb. Transgenic lines were established in the FVB genetic background by backcrossing for at least eight generations and same age and parity FVB wild type control littermates were used as control mice.

Knock in mice carrying the Ptch1 targeted disruption and lacZ marker allele (Goodrich et al., 1997) herein designated as Ptch1 lacZ, were obtained from Jackson Lab and maintained in a C57BL/6J genetic background. PCR genotyping for this allele was carried out as described (Goodrich et al., 1997). Mammary samples for analysis were obtained from the offspring of WAP Shh mice in the FVB background and *Ptch1 lacZ* mice in C57BL/6J background. WAP Shh/Ptch1 lacZ bitransgenic and Ptch1 lacZ only, same age and parity, control mammary tissues were analyzed. All experimental procedures were performed according to the European and Spanish laws and regulations and approved by our institution's ethics committee.

RNA analysis

Total RNA was isolated and Northern blots were performed as described previously (Robinson et al., 1995). The whole mouse Shh cDNA and fragments from mouse Ptch1 and WAP cDNAs were used as probes.

Histology, immunofluorescence and immunohistochemistry

Mammary tissue was harvested from recently euthanized mice. For whole mount examination, mammary tissues were fixed in Carnoy's solution and stained with carmine aluminum overnight as described previously (Kordon et al., 1995). For histological analysis, 5 μm paraffin sections were stained with H&E. For immunofluorescence, tissues were fixed with formalin and processed by standard protocols. 5 μm paraffin sections were deparaffinized and boiled in microwave oven in 10 mM Sodium Citrate Buffer, incubated overnight with primary antibodies followed by 1 h incubation with appropriate secondary antibodies.

Slides were observed using an Axio Imager A1 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) or a Leica TCS SP5 AOBS Confocal Microscope (Leica Microsystems GmbH, Wetzlar, Germany) for cilia immunofluorescence visualization.

Primary antibodies used for immunohistochemistry and immunofluorescence analysis are: Integrin Beta 3 (ITGB3) rabbit monoclonal (ab75872 Abcam) 1/100 dillution; Collagen IV (Col IV) rabbit polyclonal (ab6586 Abcam) 1/100; Collagen I (Col I) rabbit polyclonal (ab292 Abcam) 1/100 diluted; Cytokeratin 5 (K5) rabbit polyclonal (AF 138, Covance) 1/200 dilution; Cytokeratin 6 (K6) rabbit polyclonal (PRB 169P Covance) 1/100 dilution; Cytokeratin 14 (K14) rabbit polyclonal (AF64, Covance) 1/200 dilution; Cytokeratin 15 (K15) mouse monoclonal (LHK15, Thermo Fisher Scientific) 1/100 dilution; Cytokeratin 17 (K17) rabbit polyclonal (ab53707, Abcam) 1/100 dilution; Gli2 rabbit polyclonal (ab26056, Abcam) 1/100 dilution, Cytokeratin 8 (K8) rat monoclonal Troma I supernatant (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); p63 mouse monoclonal (clone 4A4, Santa Cruz) 1/20 dilution; PCNA mouse monoclonal (PC10, Abcam) 1/500 dilution; Sonic hedgehog (Shh) rat monoclonal (clone171018, Abcam) 1/20 dilution; Sonic Indian hedgehog (Shh/Ihh) rabbit polyclonal (2271, Cell Signaling) 1/50 dilution; Acetylated tubulin(ac Tub) mouse monoclonal (clone 6 11B 1, Sigma Aldrich) 1/1000 dilution; Beta galactosidase (β gal) rabbit polyclonal (55976, Cappel) 1/250 dilution; Gamma tubulin (γ Tub) rabbit polyclonal (T5192, Sigma Aldrich) 1/1000 dilution; β catenin (β cat) mouse monoclonal (clone 14, BD Transduction Laboratories) 1:100 dilution; Smooth muscle actin (SMA) clone 1A4 FITC conjugate mouse monoclonal (Sigma Aldrich) 1/250 dilution.

Immunohistochemical analysis of beta galactosidase expression was performed with the same tissue fixation, deparaffinization and antigen retrieval protocols as in the immunofluorescence analysis. However, a subsequent treatment of the tissue slides with hydrogen peroxide was needed to eliminate endogenous peroxidase activity. Serum blocking and overnight primary antibody incubation was followed by 1 h incubation with the appropriate HRP conjugated secondary antibody and 30 min with Vectastain ABC reagent (as per kit instructions). The slides were incubated for 2 min with the DAB developing solution and counterstained with hematoxylin.

Quantitative RT PCR assays

First or second lactation, number 4 mammary glands (after lymph node removal) from lactating day 1 female mice were used for RNA extractions. Glands from three different animals were used for each time point. Total RNA was isolated using Trizol Reagent (Invitrogen) according to the manufacturer's instructions, and treated with DNA Free RNA Kit (Zymo Research). cDNA was reverse transcribed from 2 μg of RNA using AMV Reverse Transcriptase (Promega). β actin was used as internal control. Primers used for β actin were: actinF 5′ TGT TAC CAA CTG GGA CGA CA 3′, actinR 5′ GGG GTG TTG AAG GTC TCA AA 3′; for Shh: ShhL 5′ ACC CCG ACA TCA TAT TTA AGG A 3′, ShhR 5′ TTA ACT TGT CTT TGC ACC TCT GA 3′; for Ptch1: Ptch1L 5′ GGA AGG GGC AAA GCT ACA GT 3′, Ptch1R 5′ TCC ACC GTA AAG GAG GCT TA 3′; for Gli1: Gli1L 5′ CTG ACT GTG CCC GAG AGT G 3′, Gli1R 5′ CGC TGC TGC AAG AGG ACT 3′; for Gli2: Gli2L 5′ GCA GAC TGC ACC AAG GAG TA 3′, Gli2R 5′ CGT GGA TGT GTT CAT TGT TGA 3′. Real time PCR was performed using the reverse transcription product as template with the GoTaq qPCR Master Mix (Promega) according to the manufacturer's instructions. Amplifications were carried out in a Light Cycler 2.0 (Roche) using the following cycles: 2 min 95 °C, 40 cycles (15 s 95 °C, 1 min 60 °C), 7 min 60 °C. The PCR products quality was checked by a melting curve analysis. Relative quantification of gene expression analysis was performed using the Pfaffl method (Pfaffl, 2001>).

Statistics

Statistical analysis was performed with Graph Pad Prism, Version 5 (Graph Pad software, Inc.). The *t* test with Welch's correction was used for unpaired data. Statistical significance was established at $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

1.1. Whole mount X gal staining of transgenic mice

For whole mount X gal staining, Ptch1 lacZ and WAP Shh/Ptch1 lacZ lactating females were used. Mammary glands from three different animals were taken for each analysis. Number 3 mammary glands were harvested on day 1 of lactation, and fixed for 2 h in 2% paraformaldehyde, 0.25% glutaraldehyde, 0.01% NP 40 in PBS. After rinsing in PBS, glands were incubated for 2 h in pre staining buffer, containing 2 mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP 40 in PBS. Then, glands were incubated at 25 °C overnight in staining buffer containing 30 mM potassium ferricyanide, 30 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP 40, and 1 mg/ml X gal in PBS. After that, half of glands were fixed in 4% paraformaldehyde and mounted in Permount. The other half were dehydrated and embedded in paraffin for sections, and stained with eosine.

Results

Generation of WAP Shh transgenic mice

To facilitate the analysis of Hh signaling in mammary gland we generated transgenic mice expressing Shh under the control of the parity inducible, mammary specific WAP gene promoter. Nine transgenic founder mice were identified by Southern blot analysis of DNA samples from mice obtained after pronuclear injection of one cell C57Bl/6JxDBA/2J F2 embryos, using a fragment of the transgene as a probe. Northern blot analysis showed expression of transgenic Shh mRNA in lactating mammary glands from lactation day 1 (L1) primiparous transgenic mice of two different lines: 119 and 186 (Fig. 1A, lanes 2 and 4). Shh expression levels in these whole organ samples were comparable to those found in embryonic E15 brain tissue, included in the Northern blot as a positive control (Fig. 1A, lane 12). Endogenous

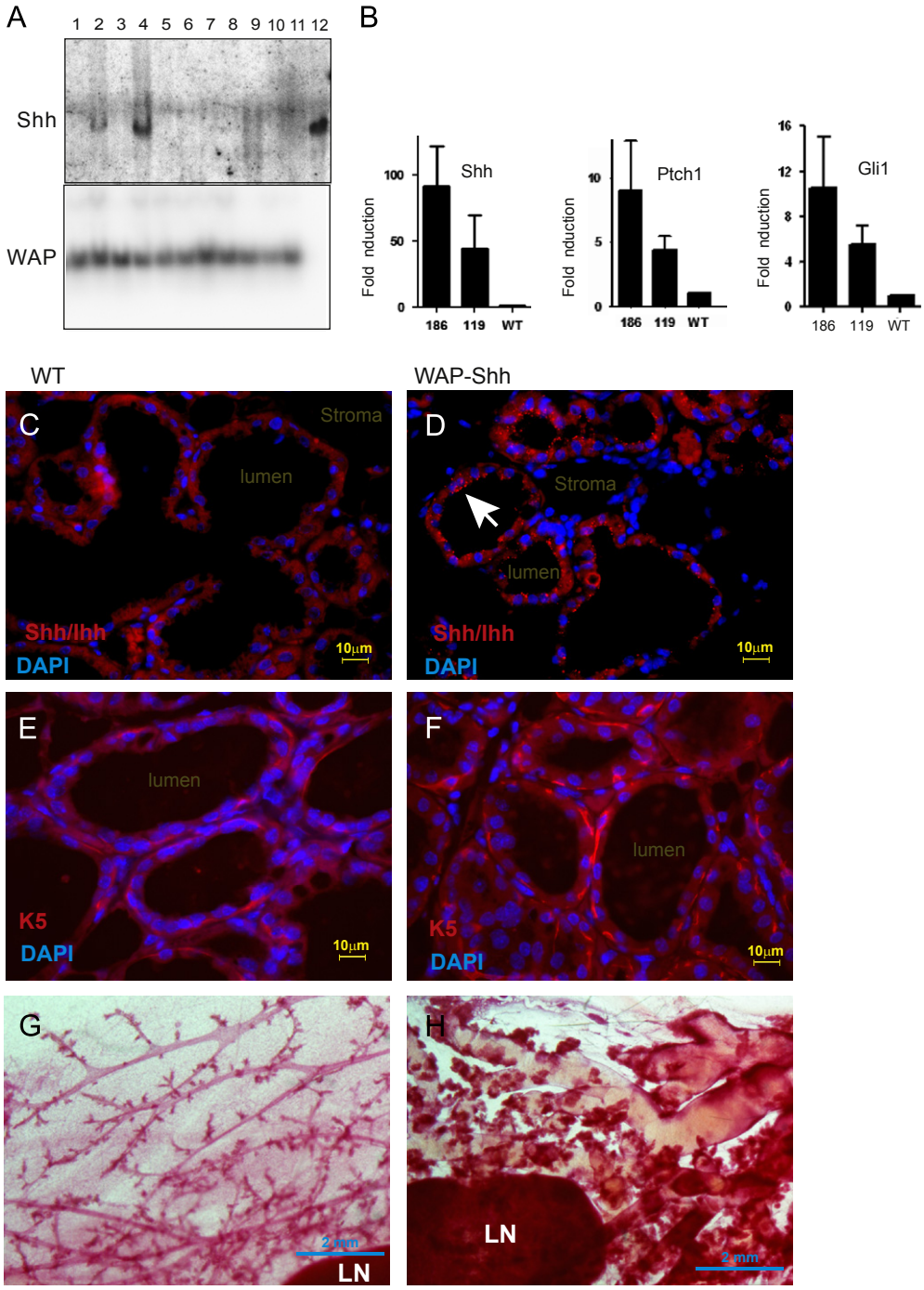

Fig. 1. Transgenic Shh expression analysis. (A) Upper panel: total RNA Northern blot analysis of Shh expression. First lactation day 1 (L1) lactating mammary tissues from transgenic lines (1–5, 7, 9–11), and wild type controls (6, 8). Wild type fetal brain E15 (positive control; 12). Lower panel: membrane reprobed with WAP as a loading control. Similar expression levels were found in all 11 samples corresponding to lactating glands (1–11). No band was detected in fetal brain control sample (12). (B) RT-qPCR analysis of Shh, Ptch1 and Gli1expression in 119 and 186 WAP-Shh transgenic lines and wild-type lactating mammary gland samples. 92 ± 29 and 44 ± 25 fold inductions of Shh expression, 9 ± 3.8 and 4.5 ± 1.0 fold inductions of Ptch1 expression and 10.5 ± 4.5 and 5.5 ± 1.6 fold inductions of Gli1 expression, relative to wild type glands (WT) is found in samples from line 186 and 119 respectively. (C and D) Immunofluorescence analysis of Hedgehog protein expression using a polyclonal antibody that detects both Ihh and Shh proteins (Shh/Ihh) in WAP-Shh (D) and wild type control (C) mammary tissue samples. (E and F) Keratin 5 (K5) in WAP-Shh (F) and wild type control (E) L1 mammary tissue samples. DAPI stained nuclei (blue). (G and H) Whole-mount analysis of 12 month-old multiparous (four lactations), involuted, mammary glands. Carmine aluminum-stained wild type (G) and WAP-Shh (H) mammary ducts from mammary gland number four. The figures show dilated ducts near the lymph node (LN) area in the WAP-Shh sample as compared to wild type control.

Shh mRNA expression was not detectable by Northern blot in wild type lactating control tissue (lanes 6 and 8).

Shh overexpression in lactating WAP Shh transgenic tissue, as compared to physiological levels in control glands, was quantified by RT qPCR analysis using samples from transgenic lines 119 and 186. We found 92 ± 29 and 44 ± 25 fold inductions of Shh expression, relative to wild type glands, in samples from line 186 and 119 respectively (Fig. 1B). Hedgehog protein expression in transgenic glands was analyzed by immunofluorescence staining using a rabbit polyclonal antibody that detects both Shh and Ihh proteins. Bright granular staining was found in the secretory epithelium of WAP Shh transgenic glands (Fig. 1D, arrow), but

not in control mice glands (Fig. 1C). Mice from the two transgenic Shh expressing lines (henceforth referred to as WAP Shh mice) were used in subsequent experiments.

To assess the extent of Hh pathway activation in transgenic mammary glands, we analyzed the expression of Ptch1 and Gli1, both Hh target genes and well established indicators of pathway activity. RT qPCR experiments showed Ptch1 and Gli1 induction in the two transgenic lines. Ptch1 induction was 9 ± 3.8 and 4.5 ± 1.0 fold, relative to wild type mammary glands, and Gli1 induction was 10.5 ± 4.5 and 5.5 ± 1.6, in samples from lines 186 and 119 respectively (Fig. 1B). These data prove at a molecular level the activation of the Hh pathway in WAP Shh mammary glands.

The overall structure of the mammary glands was analyzed by preparing whole mount stainings of WAP Shh and control littermate samples. Morphological abnormalities consisting of ductal dysplasias and ectasia in not lactating mammary glands from multiparous (four lactation cycles) mice were obvious in WAP Shh samples (Fig. 1H) but not in wild type (WT) same parity control littermates (Fig. 1G). These phenotypic alterations were suggestive of the presence of a cellular population in which activation of the Hh pathway in response to transgenic Shh expression resulted in altered growth or differentiation. In addi tion, immunofluorescence analysis of basal cell marker keratin 5 showed remarkably increased expression in transgenic (Fig. 1F) as compared to control lactating mammary gland sections (Fig. 1E), also suggesting altered mammary gland differentiation.

Although a fraction of aging (older than 18 months) WAP Shh mice in the FVB genetic background developed mammary tumors, a similar incidence of mammary tumorigenesis was found in non transgenic control littermates. It has been previously described that spontaneous tumorigenesis occurs in mammary tissue of aging FVB mice (Radaelli et al., 2009). However, no mammary tumors were found when WAP Shh mice where bred into other genetic backgrounds (DBA/2) or into the hybrid background in which our model is established (FVB x C57Bl/6) F1. For this reason, we did not characterize WAP Shh tumors further.

Detection of Hh responsive cells in the mammary tissue of WAP Shh mice

To detect Hh responsive cells, we analyzed the Hedgehog pathway activity in the mammary tissue in situ by generating mice that carry both the WAP Shh transgene and a Patched 1 lacZ reporter allele (Ptch1 lacZ), in which the expression of a nuclear targeted β gal protein is driven by the endogenous Ptch1 gene regulatory region (Goodrich et al., 1997). This marker knock in allele is also null for the Ptch1 gene and results, in itself, in limited activation of the Hh pathway due to Ptch1 haploinsufficiency (Lewis et al., 1999). To obtain these double transgenic mice, we bred WAP Shh transgenic mice, previously backcrossed into the FVB genetic background, with Ptch1 lacZ mice in C57Bl/6J genetic background. F1 offspring female mice were used for mammary gland analysis. β gal activity was analyzed at lactation day 1(L1) to allow for highest WAP promoter expression. Mammary whole mount X gal staining revealed some limited, scattered areas with detectable β gal activity in WAP Shh/Ptch1 lacZ samples from first lactation, but staining was more widespread and readily detectable in second lactation samples (Fig. 2C, arrow). Consis tently, RT qPCR analysis allowed us to quantify in 2.5 ± 0.4 and 2.5 ± 0.2 fold the increase of Ptch1 expression between second and first lactation, in lines 186 and 119 respectively (Fig. 2A). Sections of X gal staining are shown in Fig. 2D G. No staining was found in Ptch1 lacZ control littermates mammary sample pre parations (Fig. 2B and D).

Immunohistochemical detection of the β gal protein was performed to identify the Hh responsive cells in paraffin sections of WAP Shh/Ptch1 lacZ lactating mammary tissues. We detected clusters of mammary epithelial cells strongly immunoreactive for β gal protein with a distinct nuclear staining pattern, revealing the sites where the Hh pathway was active (Fig. 2H, magnified in I, black arrow). The rest of mammary epithelial cells in the gland presented a faint immunostaining for β gal (Fig. 2I, blue arrow) while the stroma was clearly negative (Fig. 2I, red arrow). No immunostaining for β gal was found in mammary tissue from control mice not carrying the lacZ allele (not shown).

β gal positive cells were found preferentially in a basal loca tion in dysplastic areas of ducts and lobules. Despite their basal location, these Ptch1 expressing cells did not have the flat appearance of bona fide myoepithelium, resembling a cuboidal epithelium instead (Fig. 2I, black arrow).

To analyze the correlation between transgenic Shh expression and Hh responding cells, we performed double immunofluores cence staining in paraffin sections of lactating mammary glands from WAP Shh/Ptch1 lacZ bitransgenic and Ptch1 lacZ control mice to simultaneously detect β gal and Shh proteins. Using a monoclonal antibody that specifically detects Shh, we observed areas of robust Shh staining in mammary luminal cells of bitransgenic mice, as expected for a WAP driven transgenic protein (Fig. 2K, arrow). Noteworthy, β gal staining, revealing endogenous Ptch1 expression, was found exclusively in adjacent, basally located cells, but not in the luminal, WAP expressing cells where the Shh transgene is expressed. No staining was found for Shh or β gal in Ptch1 lacZ control tissue (Fig. 2J).

To characterize these Hedgehog target cells in the mammary tissue, we simultaneously analyzed keratin 8 (K8), a marker for luminal cells, and β gal expression. β gal expressing cells did not express K8, confirming their non luminal identity (Fig. 2M), and were arranged as a single layer, occasionally multilayer, of cells placed in a basal location with respect to keratin 8 positive luminal cells (Fig. 2M, white arrow). The apparent occasional detection of K8 in sections of multilayered basal epithelium (Fig. 2M, yellow arrow) is in fact produced by K8 expression in a lower plane, and can be observed because β gal positive cells are small and do not form a continuous layer. No staining for β gal was found in Ptch1 lacZ control samples (Fig. 2L).

We confirmed the luminal identity of Hh expressing cells in our model by double Shh Ihh/K8 immunofluorescence analysis of WAP Shh/Ptch1 lacZ and Ptch1 lacZ control lactating mammary tissues. We detected strong Hh expression in luminal (K8 positive cells) in WAP Shh/Ptch1 lacZ as compared to faint expression in Ptch1 lacZ control lactating mammary glands (supplementary Fig. S1).

These data point to a paracrine mode of action where Shh protein, secreted by luminal epithelium, induces Ptch1 expres sion, and therefore Hh pathway activation, in adjacent cells, but not in the Shh producing cells themselves.

Hh responsive cells overexpressed basal markers and lacked SMA expression

Since Ptch1 expressing, β gal positive cells were located in a basal position, we decided to analyze whether they expressed mammary myoepithelial cell markers keratin 5 (K5), keratin 14 (K14), keratin 17 (K17), p63 and smooth muscle actin (SMA). Double staining showed that β gal expression and high expres sion levels of p63 co localized in the dysplastic areas found in WAP Shh/Ptch1 lacZ mammary tissue (Fig. 3F), while normal myoepithelial cells found in Ptch1 lacZ control samples displayed low p63 staining and did not present detectable β gal staining (Fig. 3C, arrow). Double immunofluorescence analysis for basal

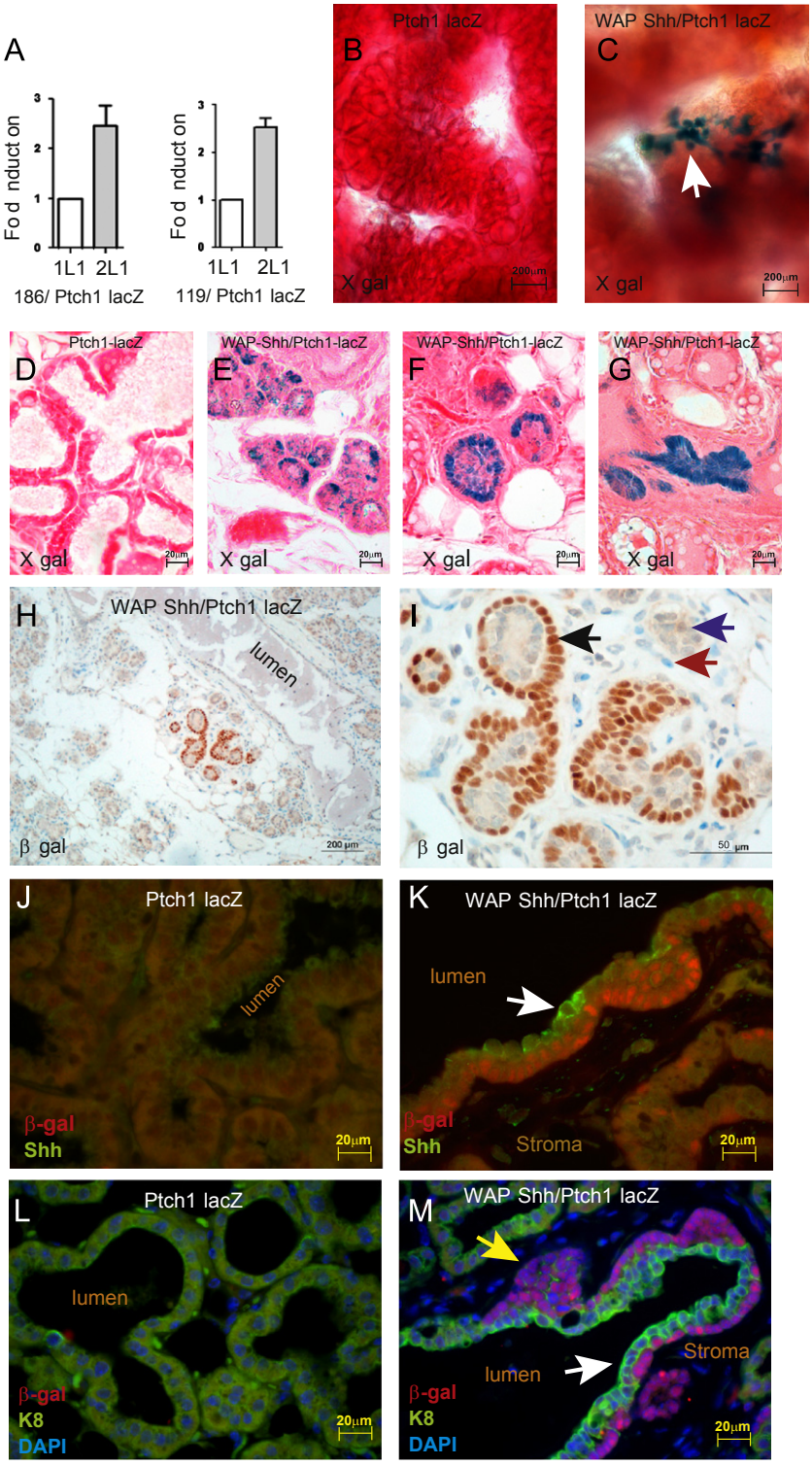

Fig. 2. Detection of Hh-responsive cells in mammary tissues of WAP-Shh/Ptch1-lacZ mice. (A) Fold increase of Ptch1 expression in second vs. first lactation. RT-qPCR analysis of Ptch1 expression in 119 and 186 WAP-Shh/Ptch1-lacZ lines, mammary tissue samples, in the first day of first (1L1) and first day of second (2L1) lactation. (B and C) Whole-mount preparations of Ptch1-lacZ control (B) and WAP-Shh/Ptch1-lacZ (C) second lactation mammary gland samples subjected to X-gal staining. (D–G) Paraffin sections of X-gal-stained and eosin-counterstained whole-mount preparations of Ptch1-lacZ (D) and WAP-Shh/Ptch1-lacZ (E, F, G) second lactation mammary gland samples. Blue X-gal staining was detectable in mammary samples from line 119 WAP-Shh/Ptch1-lacZ (E) and line 186 WAP-Shh/Ptch1-lacZ (F and G), while no staining was found in Ptch1-lacZ control (D). (H) Immunohistochemical analysis of β-gal protein on a paraffin section of a second lactation mammary tissue sample from a WAP-Shh/Ptch1-lacZ mouse. β-gal-positive alveoli (brown nuclei) can be observed near a wide duct (lumen). (I) Higher magnification of the same area. β-gal-positive cells constitute the outer layer of dysplastic alveoli (black arrow). Faint β-gal staining (blue arrow) in non-dysplastic alveoli. Stromal cells are β-gal-negative (red arrow). (J and K) β-gal/Shh immunofluorescence detection in a WAP-Shh/Ptch1-lacZ dysplastic (K) and Ptch1-lacZ control (J) mammary tissues taken from fourth lactation day 3 mammary glands. A continuous monolayer, occasionally multilayer, of β-gal-positive cells in a basal location (K, red nuclei) and strong Shh staining in some of the luminal cells neighboring β-gal-positive cells (K, arrow) are shown. β-gal and Shh are not detectable in Ptch1-lacZ control tissue (J). (L and M) β-gal/K8 immunofluorescence analysis of sections from Ptch1-lacZ control (L) and WAP-Shh/Ptch1-lacZ dysplastic (M) fourth lactation mammary tissues. K8 marks luminal cells. β-gal-positive cells are only detectable in M and constitute the outer layer of a mammary duct. K8-positive cells are β-gal negative (M, white arrow). β-gal positive nuclei (red) surrounded by green fluorescence staining (yellow arrow) are shown. K8-positive cells (green), in a lower plane, can be seen through round and small β-gal-positive cells that do not form a continuous layer.

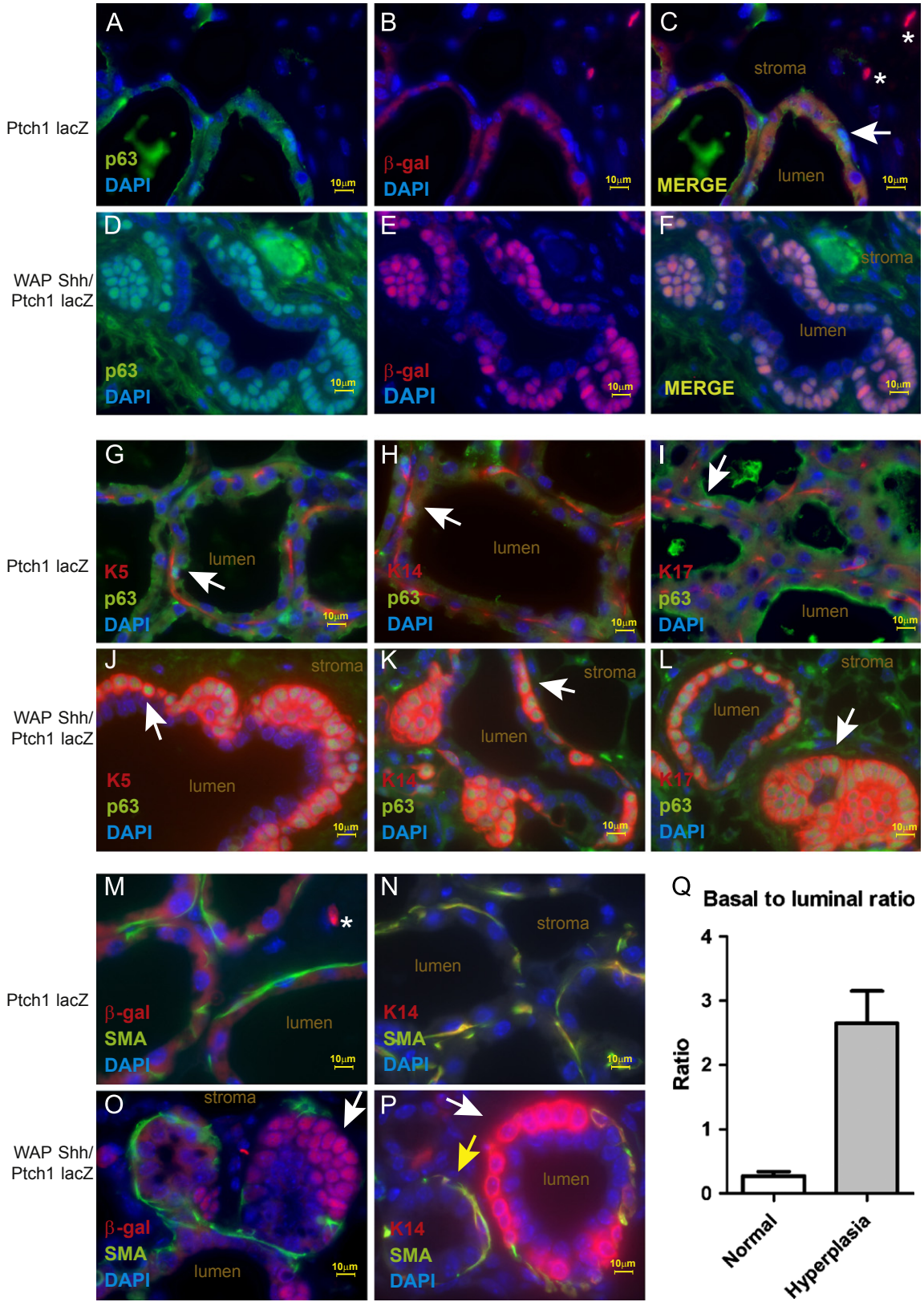

Fig. 3. Hh-responsive cells overexpress basal markers and lack SMA expression. Immunofluorescence analysis of basal makers expression in WAP-Shh/Ptch1-lacZ transgenic (D–F, J–L, O and P) and Ptch1-lacZ control (A–C, G–I, M and N) fourth lactation day 1 mammary tissue sections. (A–F) β-gal/p63 detection in Ptch1-lacZ control (A–C) and WAP-Shh/Ptch1-lacZ samples (D–F). p63 positive/β-gal negative nuclei in normal myoepithelial cells (C, arrow) and p63 negative/β-gal positive nuclei in perineural fibroblasts at peripheral nerves (C, asterisks) in Ptch1-lacZ control samples. p63 and β-gal colocalize in nuclei of basal cells in dysplastic WAP-Shh/Ptch1-lacZ mammary epithelium (F). (G–L) Overexpression of p63 and K5, K14 and K17 keratins in WAP-Shh/Ptch1-lacZ basal hyperplasias (J–L, arrows) and co-expression but no overexpression in Ptch1-lacZ control myoepithelium (G-I, arrows). (α-p63 mouse monoclonal antibody yielded some non-specific cytoplasmic and extracellular staining). (N–P) β-gal/SMA (M and O) and K14/SMA (N and P) expression in Ptch1-lacZ control and dysplastic lesions in WAP-Shh/Ptch1-lacZ samples. β-gal positive (O) and K14-overexpressing basal epithelial cells (P) in WAP-Shh/Ptch1-lacZ dysplastic lesions do not express SMA (O and P, white arrows). SMA is readily detectable in WAP-Shh/Ptch1-lacZ normal alveoli (P, yellow arrow) or Ptch1-lacZ control mammary tissues (M and N). The asterisks in M marks a β-gal positive nuclei in a perineural fibroblasts at a peripheral nerve. (Q) Basal to luminal cell ratio in fourth lactation WAP-Shh/ Ptch1-lacZ dysplastic and Ptch1-lacZ control mammary tissues. A 10-fold expansion of the basal compartment was found in WAP-Shh/ Ptch1-lacZ Hh-signaling areas: 2.65 ± 0.57 basal/luminal ratios in WAP-Shh/Ptch1-lacZ dysplastic areas vs. 0.28 ± 0.06 basal/luminal ratio in lactating Ptch1-lacZ control tissue ($p < 0.001$).

keratins and p63 expression showed that cells overexpressing p63 also overexpressed K5, K14 and K17 in WAP Shh/Ptch1 lacZ mice (Fig. 3J L) therefore implying that overexpression of basal keratins correlated with Ptch1 expression. We could not directly perform double staining for β gal and basal layer keratins since all available antibodies amenable to this staining strategy were produced in rabbit. Ptch1 expressing cells in dysplastic areas were therefore characterized by very high expression of basal markers and a cuboidal morphology, clearly differentiating them from flat shaped myoepithelial cells that also expressed basal keratins, albeit at much lower levels (Fig. 3G I).

We next performed double immunofluorescence analysis to study the presence of smooth muscle actin (SMA) in β gal positive cells. In contrast to the basal markers tested previously, SMA was totally absent from the dysplastic regions, showing the fact that β gal and SMA expression were mutually exclusive (Fig. 3O, arrow). Consistently, double immunostaining for SMA and K14 showed absence of SMA expression in K14 overexpressing cells in basal hyperplasias found in WAP Shh/Ptch1 lacZ mammary glands (Fig. 3P, white arrow and supplementary Fig. S2A, arrow) but not in normal myoepithelial cells present in WAP Shh/Ptch1 lacZ (Fig. 3P, yellow arrow) or Ptch1 lacZ mammary myoepithelium (Fig. 3N). We also demonstrated that K14 overexpressing cells do not express luminal marker K8 (Supplementary Fig. S2B, arrow).

Additional panels showing more extended areas of the dysplastic basal epithelium are shown in supplementary Fig. S3B D.

In addition to the increased expression of basal markers in the Hh responsive cells, we found a significant increase of the basal to luminal cell ratio in WAP Shh/Ptch1 lacZ Hh signaling areas (2.65 ± 0.5) compared to normal lactating Ptch1 lacZ control tissue (0.28 ± 0.06; $p < 0.001$). This 9.5 fold increase of basal compartment suggests that basal cell hyperplasia is in the origin of WAP Shh/Ptch1 lacZ mammary glands dysplasias. Despite the presence of basal hyperplasia and dysplasias, no mammary tumors were found in WAP Shh/Ptch1 lacZ mice, or in non transgenic littermates, in this genetic background.

Hedgehog pathway activation caused morphological alterations of mammary tissues

Histological analysis of lactating mammary tissues from WAP Shh/Ptch1 lacZ bitransgenic mice revealed dysplastic areas extending along ducts and lobules (Fig. 4B, magnified in D). No morphological alterations were apparent at this stage in Ptch1 lacZ control littermates glands (Fig. 4A). The dysplastic areas presented scarce lobulo alveolar structures some of which contained an intensely stained secretion (Fig. 4B, black arrow) different from the milk shown in control lactating Ptch1 lacZ tissue (Fig. 4A, black arrow). Protruding from ducts, there were solid hemispherical structures composed of small round cells with clear nucleoplasm (Fig. 4D, green arrow). β gal immunofluorescence analysis of the same area shown in Fig. 4B, showed positive β gal nuclear staining in all cuboidal shaped basal layer cells (Fig. 4C, yellow arrow) as well as in the cells that formed the characteristic solid structures (Fig. 4C white arrow), demonstrating the coincidence of Hh pathway activation with dysplastic areas.

We examined Wnt pathway activation in dysplastic areas since activation of this pathway by Hh signaling has been shown in other organs (Shin et al., 2011) and Wnt activation is commonly associated with dysplastic lesions in mouse mammary tumor models (Gallego et al., 2003; Miyoshi et al., 2002). Double immunofluorescence staining for β catenin and β gal showed that β catenin was localized exclusively at the cellular membrane, and not in the cytoplasm or nuclei, in Shh responding or adjacent cells (Fig. 4C), indicating that the Wnt pathway is not active in Hh driven mammary lesions.

In addition to the epithelial changes found in Hh responsive areas, a thicker stroma was observed around the hyperplastic structures (Fig. 4B, blue arrow), as compared to stroma near normal alveoli (Fig. 4A, blue arrow). Double immunofluorescence detection of collagen I (Col I), a main component of the extracellular matrix, and myoepithelium marker SMA, showed increased Col I deposition around Hh associated hyperplasias, identified by the absence of SMA expression (Fig. 4F, white arrow) as compared to the thin Col 1 deposition around normal alveoli in Ptch1 lacZ lactating tissue (Fig. 4E arrow). Some Col I could be detected between the epithelial cells of the hyperplasia (Fig. 4F, yellow arrow). This suggests that hyperplastic basal layer cells are not forming a continuous layer.

Expression of collagen IV, a component of the basement membrane produced and secreted by myoepithelial and mesenchymal cells (Warburton et al., 1986) was analyzed to further characterize Hh responding cells. Double immunofluorescence detection of collagen IV and p63, a surrogate marker of Hh activation in our model, showed that while in normal ducts and alveoli, found in Ptch1 lacZ control littermates mammary glands, collagen IV was localized at the basement membrane forming a lamina between the epithelium and the stroma (Fig. 4G), in hyperplastic ducts and lobules of WAP Shh/Ptch1 lacZ mammary glands, Col IV is overexpressed and it completely encircled p63 overexpressing cells (Fig. 4H, arrow). This pattern of collagen IV deposition is further proof of the altered differentiation caused by Hh activation in basal mammary epithelial cells.

Nuclear localization of Gli2 protein in Hh responding cells and increased expression of Gli1 and Gli2 genes in WAP Shh/Ptch1 lacZ mammary tissue

To further characterize Hh pathway activation in the hyperplastic regions adjacent to Shh expressing cells found in bitransgenic mammary tissue, we performed double immunofluorescence staining for p63 and Gli2 transcription factor, the initial executor of Hh induced transcriptional activation. We found nuclear Gli2 staining in p63 overexpressing cells (Fig. 5D F) while only cytoplasmic staining was detected in control Ptch1 lacZ lactating tissue samples (Fig. 5B). These data show functional activation of the Hh pathway in the basal cell hyperplastic areas.

To quantitatively assess pathway induction in transgenic tissues, we performed RT qPCR analysis of Gli1 and Gli2 expression. We found that both Gli1 and Gli2 transcripts were induced in WAP Shh/Ptch1 lacZ lactating mammary tissues as compared to wild type controls (Fig. 5G). Gli1showed fold inductions 21.2 ± 3.8 and 6.7 ± 0.3 relative to wild type samples, in lines 186 and 119 respectively and Gli2 showed fold inductions 20.5 ± 12.0 and 6.2 ± 2.0 relative to wild type samples, in lines 186 and 119, respectively. Some induction of Gli1 and Gli2 was also found for Ptch1 lacZ control tissues relative to wild type samples, although at remarkably lower levels than in the double transgenic tissues (2.75 ± 0.1 fold induction for Gli1 and 4.6 ± 1.1 fold induction for Gli2 (Fig. 5G)).

Expression of mammary progenitor cell markers in the hyperplastic basal compartment of WAP Shh/Ptch1 lacZ mammary glands

Mammary progenitor and stem cells have been characterized by their integrin receptors expression pattern (Shackleton et al., 2006; Stingl et al., 2006). Specifically, integrin β3 (Itgb3), has been identified as a marker for the luminal progenitor compartment

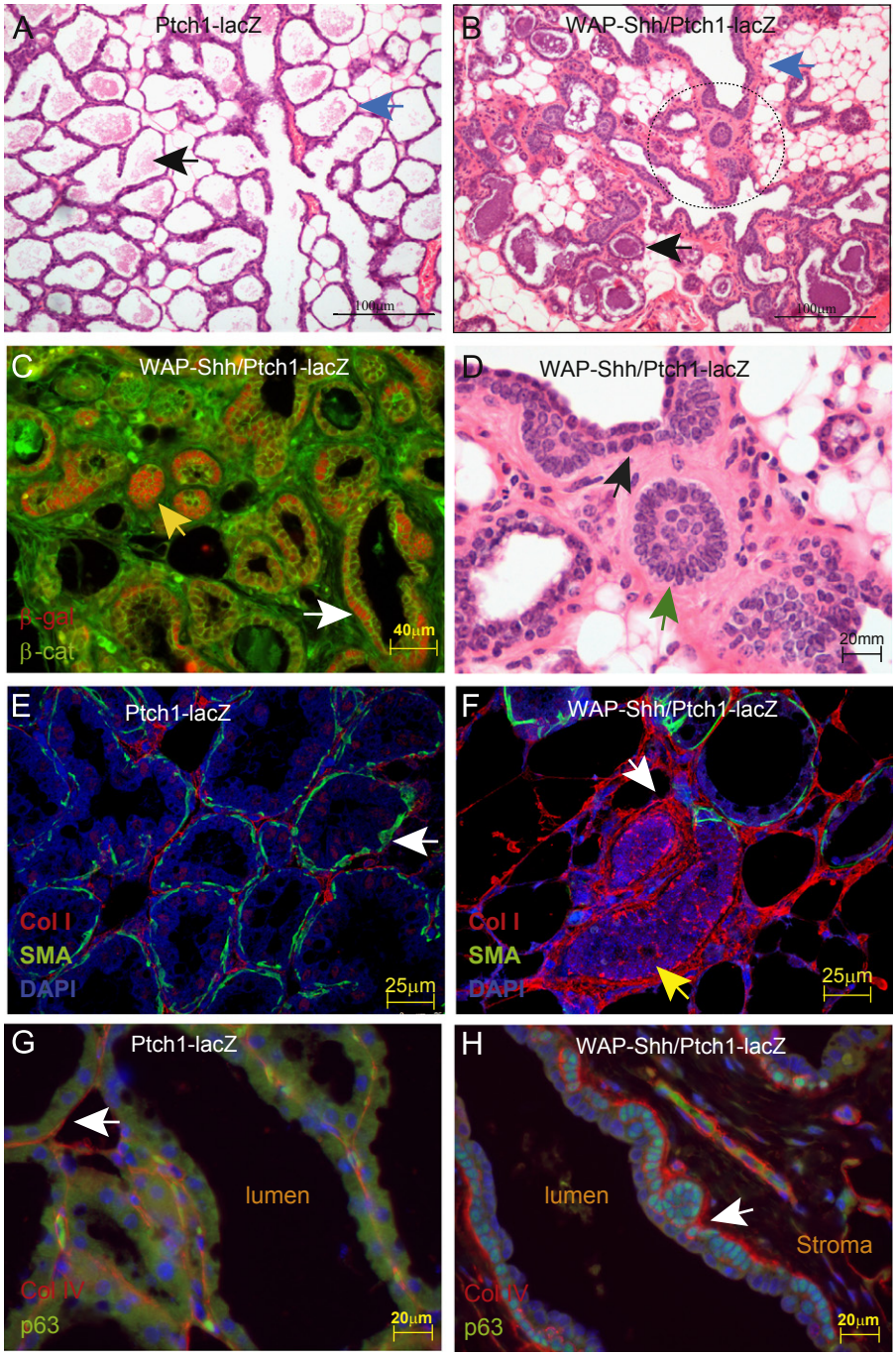

Fig. 4. Hedgehog pathway activation causes morphological alterations in WAP-Shh/Ptch1-lacZ mammary tissues. Histological and immunofluorescence analysis of fourth lactation mammary tissue samples. (A and B) Histological analysis of Ptch1-lacZ control (A) and dysplastic WAP-Shh/Ptch1-lacZ (B) mammary samples. Lactating tissue displays expanded lobulo-alveolar structures filled with milk (A, black arrow) surrounded by mammary fat-pad (A, blue arrow). Altered lobulo-alveolar structure containing intensely stained secretion different from milk (B, black arrow) and spherical solid structures surrounded by a thick stroma (B, blue arrow). (C) β-gal/β-catenin immunofluorescence detection of the same area as shown in B. β-gal-positive cells are present in solid spherical structures (white arrow) and abnormal cuboidal-shaped basal epithelium (yellow arrow). β-catenin is detected exclusively at the cell membrane both in β-gal positive and negative cells. (D) A higher magnification of the histological preparation shown in B (highlighted area). Cuboidal-shaped basal epithelium (black arrow) and abnormal spherical solid epithelial structure (green arrow). The stroma is thick around hyperplastic structures. (E and F) Confocal laser scanning immunofluorescence detection of Collagen I (Col I) in Ptch1-lacZ control tissue (E) and WAP-Shh/Ptch1-lacZ hyperplasias (F). Col I/SMA immunofluorescence detection showing normal expanded alveoli expressing SMA and surrounded by a thin layer of Col I (E, arrow). Hyperplastic structures lacking SMA expression surrounded by a thicker layer of Col I (F, white arrow). Col I expression between hyperplastic epithelial cells (4F, yellow arrow). (G and H) p63/Col IV detection showing Ptch1-lacZ control (G) and WAP-Shh/Ptch1-lacZ hyperplastic (H) ducts. Collagen IV is located at the basement membrane in G (arrow) and it is overexpressed and encircles p63-overexpressing cells in H (arrow).

(Asselin Labat et al., 2007). We studied the expression of integrin β3 in WAP Shh/Ptch1 lacZ and Ptch1 lacZ control mammary tissue sections by performing double immunofluorescence staining for integrin β3 and p63 (a marker for Hh responsiveness in our model). Normal, non hyperplastic areas of transgenic lactating tissue and wild type control displayed integrin β3 staining only in isolated cells, most of which did not express p63 (Fig. 6A, arrow). A different expression pattern was found in the hyperplastic areas of bitransgenic mammary tissue, where abundant integrin β3 expression was detected in clusters of

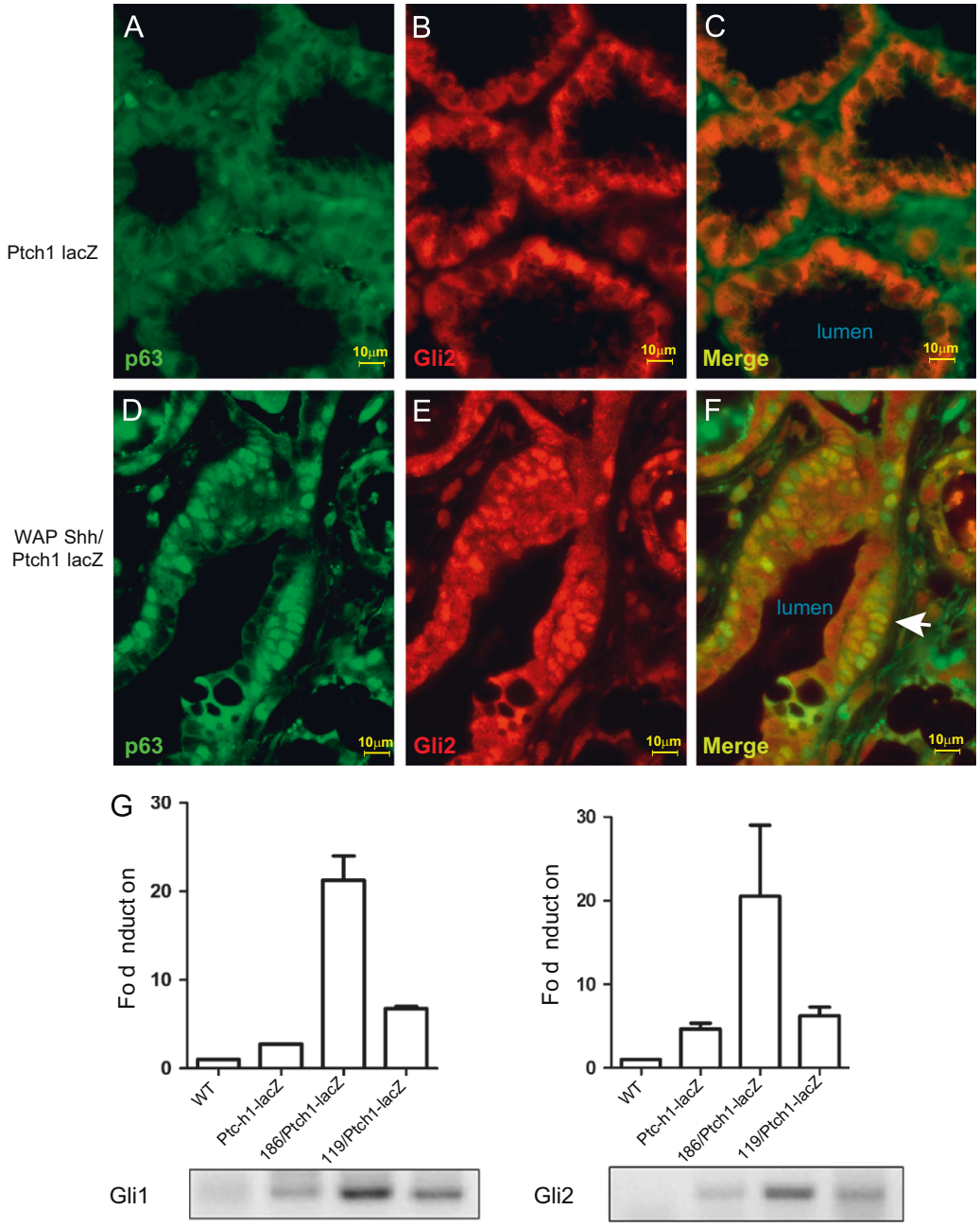

Fig. 5. Nuclear localization of Gli2 protein in Hh-responding cells and increased expression of Gli1 and Gli2 genes in WAP-Shh/Ptch1-lacZ mammary tissue. (A–F) p63/Gli2 immunofluorescence analysis of paraffin sections from fourth lactation of Ptch1-lacZ control (A–C) and hyperplastic WAP-Shh/Ptch1-lacZ (D–F) mammary tissue: p63 expression in the nuclei of hyperplastic basal epithelium (D); Gli2 nuclear localization in hyperplastic basal epithelium (E); p63 and Gli2 nuclear colocalization (F). (G) RT-qPCR analysis of Gli1 (left) and Gli2 (right) expression in wild-type (WT), Ptch1-lacZ control, 119 and 186 WAP-Shh/Ptch1-lacZ transgenic lines lactating tissue. Gli1shows fold inductions 21.2 ± 3.8 and 6.7 ± 0.3 and Gli2 shows fold inductions 20.5 ± 12.0 and 6.2 ± 2.0 in lines 186 and 119 relative to WT tissue samples. A fold induction 2.75 ± 0.1 for Gli1 and 4.6 ± 1.1 fold induction for Gli2 expression is found for Ptch1-lacZ control tissues relative to WT samples. Lower panels: Gli1 and Gli2 RT-PCR products (ethidium bromide-stained agarose gel).

Hh responding, p63 positive cells (Fig. 6D, arrow). Quantification of the percentage of integrin β3 positive cells revealed a 55 fold increase in the number of cells expressing this progenitor cell marker in dysplastic areas of lactating WAP Shh/Ptch1 lacZ mammary tissue, relative to lactating Ptch1 lacZ control tissue ($28 \pm 1.2\%$ vs. $0.5 \pm 0.02\%$; Fig. 6G).

Expression of another luminal progenitor marker, keratin 15 (K15), not usually present in normal mouse mammary tissues (Mikaelian et al., 2006), was also found in clusters in Hh responsive, β gal positive cells (Fig. 6E, arrow), while only weak and scattered K15 staining could be found in Ptch1 lacZ control (Fig. 6B, arrow).

Clusters of integrin β3 expressing cells were more abundant and overlapped to some extent, but not completely, with cells expressing keratin 15 in WAP Shh/Ptch1 lacZ dysplastic areas (Fig. 6H, arrow).

Expression of keratin 6 (K6), that is only expressed at sig nificant levels in the mouse mammary gland during pregnancy, but not during lactation, and is also considered a progenitor cell marker (Smith et al., 1990), was occasionally found in discrete clusters of cells (2 8 cells) adjacent to K15 expressing cells (Fig. 6F, arrow) in WAP Shh/Ptch1 lacZ dysplastic areas, while it was absent in Ptch1 lacZ control samples (Fig. 6C). These K6 positive cells were p63 negative, suggesting that K6 expression

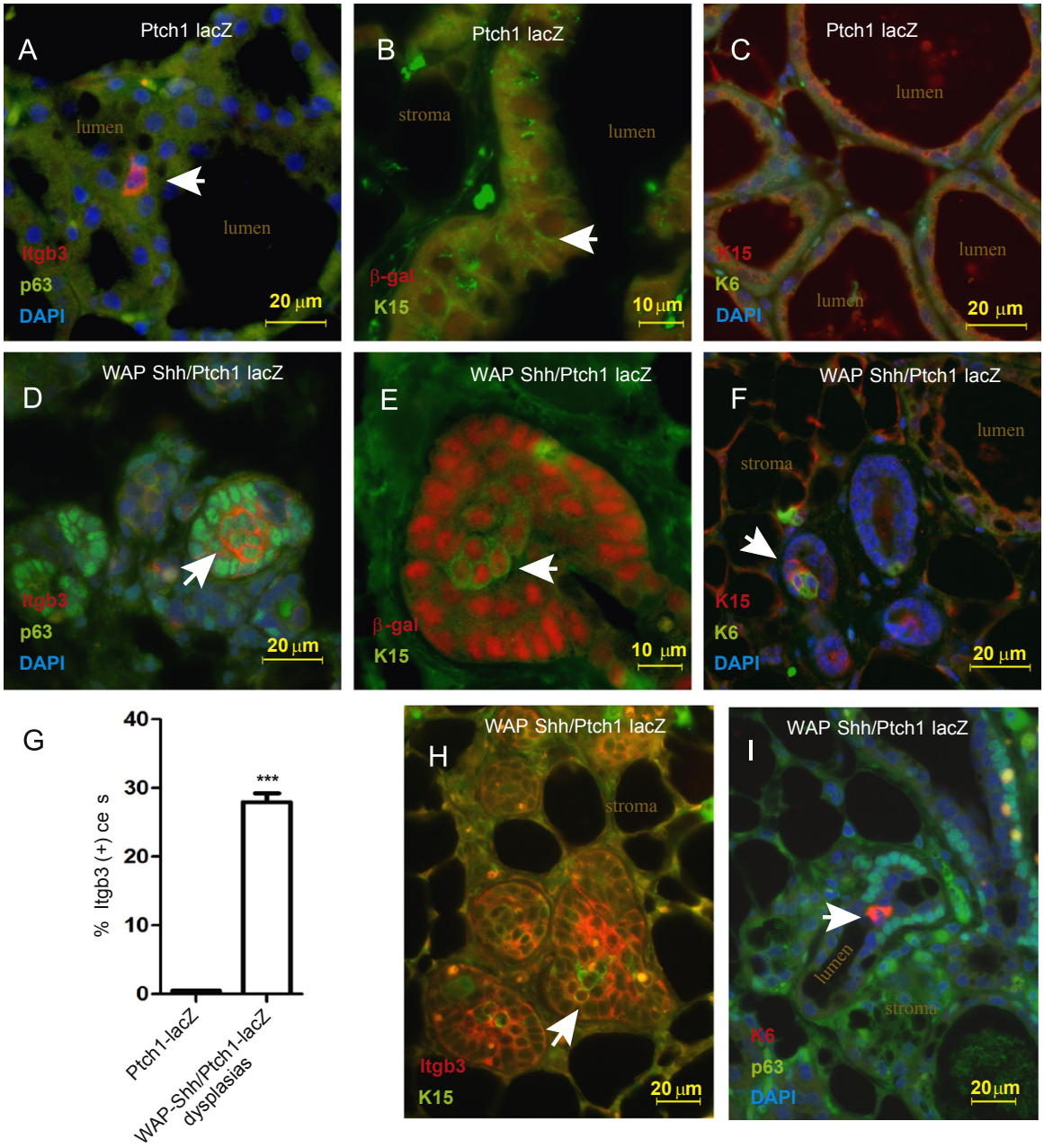

Fig. 6. Progenitor cell markers expression in dysplastic areas of WAP-Shh/Ptch1-lacZ lactating mammary glands. (A–F, H and I) Progenitor cell markers immunofluorescence detection in Ptch1-lacZ (A–C) and WAP-Shh/Ptch1-lacZ dysplastic (D–F, H and I) second (A and D) or fourth lactation (B, C, E, F, H and I) mammary tissues. (A and D) p63/integrin β3 staining: integrin β3 (Itgb3) is detected in scarce, isolated, p63 negative, luminal cells in Ptch1-lacZ control samples (A, arrow) and abundant integrin β3 expression is found in clusters of p63-positive cells in WAP-Shh/Ptch1-lacZ dysplastic regions (D, arrow). (B and E) K15/β-gal staining: β-gal-negative cells in Ptch1-lacZ control samples display weak and scattered K15 staining (B, arrow) and K15 expression is readily detectable in clusters of β-gal-positive cells in WAP-Shh/Ptch1-lacZ dysplastic regions (E, arrow α-K15 mouse monoclonal antibody yielded some non-specific stromal staining). (C and F) K15/K6 staining: K6 expression is absent in Ptch1-lacZ control samples (C) and K6-expressing cells adjacent to K15-expressing cells in WAP-Shh/Ptch1-lacZ dysplastic region (F, arrow). (G) Percentage of integrin β3 cells in Ptch1-lacZ mammary tissue and WAP-Shh/Ptch1-lacZ mammary hyperplasias at lactation day 3 showing a 55-fold increase in Itgb3 (+) cells in dysplastic areas of lactating WAP-Shh/Ptch1-lacZ mammary tissue, relative to lactating Ptch1-lacZ control tissue ($28 \pm 1.2\%$ vs. $0.5 \pm 0.02\%$). H) integrin β3/K15 expression. Integrin β3-expressing and K15-expressing cells partially overlap in a limited co-expression area (arrow). (I) p63 and K6 staining: K6 expression is found in scarce luminal cells that do not express p63 but are adjacent to p63-overexpressing cells (arrow).

is not a direct target of the Hh pathway, but might be induced in cells adjacent to Hh responding cells (Fig. 6I, arrow).

Hh responding cells in WAP Shh/Ptch1 lacZ dysplastic areas present a low proliferation rate

Immunofluorescence staining for PCNA showed a lower prolif eration rate in WAP Shh/Ptch1 lacZ Hh signaling areas, revealed by β gal and basal marker K14 overexpression (Fig. 7F, H), as compared to proliferation in Ptch1 lacZ control tissue (Fig. 7E, G). A two fold decrease in PCNA incorporation was found in WAP Shh/Ptch1 lacZ Hh signaling areas than in Ptch1 lacZ control lactating tissue. ($5.2\% \pm 0.9781$ vs. $10.76\% \pm 0.5367$; Fig. 7A). Furthermore, within the dysplastic region PCNA incorporation was five times lower in Hh responsive, β gal(+) cells, than in adjacent β gal( ) cells. The PCNA(+) β gal( ) cells/PCNA(+)β gal(+) cell ratio was

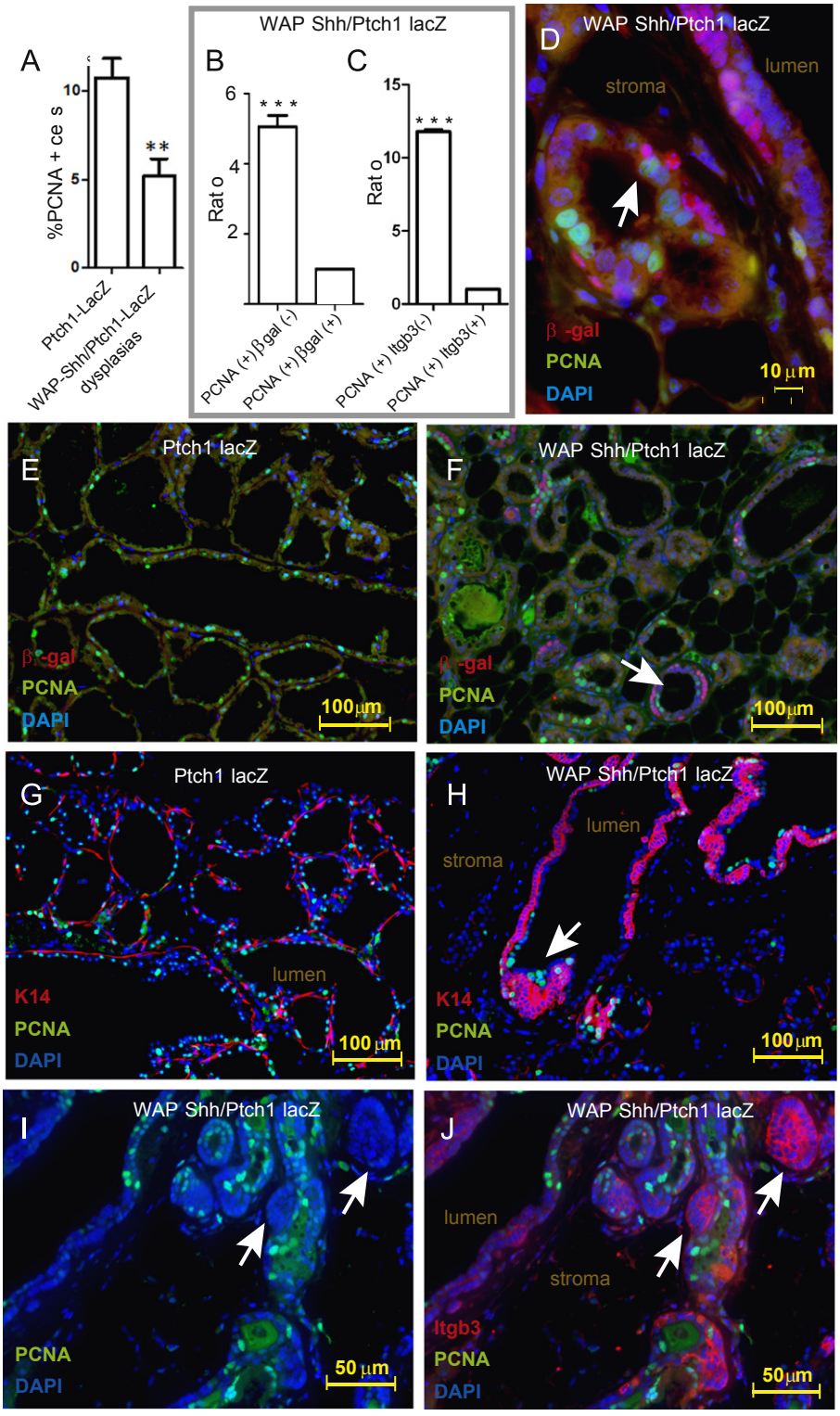

Fig. 7. Hh-responsive cells in WAP-Shh/Ptch1-lacZ dysplastic areas present a low proliferation rate. (A) Percentage of PCNA incorporation in Ptch1-lacZ control and WAP-Shh/Ptch1-lacZ dysplastic areas from fourth lactation mammary tissues. PCNA incorporation rate is $5.2 \pm 0.97815\%$ in WAP-Shh/Ptch1-lacZ dysplastic regions vs. $10.76 \pm 0.5367\%$ in Ptch1-lacZ control tissue. (B) PCNA(+)/ β-gal(−) to PCNA(+)/β-gal(+) cell ratio within dysplastic areas in WAP-Shh/Ptch1-lacZ fourth lactation day 1 mammary tissue is 5.1 ± 0.6 to 1. (C) PCNA(+)/integrin β3(−) to PCNA(+)/integrin β3(+) cell ratio within dysplastic areas in WAP-Shh/Ptch1-lacZ fourth lactation day 1 mammary tissue is 11.88 ± 0.1 to1. (D–J) PCNA immunofluorescence detection on Ptch1-lacZ control (E and G) and hyperplastic WAP-Shh/Ptch1-lacZ (D, F, H–J) fourth lactation day 1 mammary tissues. (D–F) β-gal/PCNA immunofluorescence detection. (G and H) K14/PCNA immunofluorescence detection. Arrows in D, F and H show PCNA-positive luminal cells adjacent to PCNA-negative hyperplastic basal epithelium. (I and J) PCNA and β3-integrin staining. Clusters of PCNA negative cells (I, arrow) are β3-integrin positive (J, arrow; α-PCNA mouse monoclonal antibody yielded some non-specific cytoplasmic staining).

5.1 ± 0.6 1 (Fig. 7B, illustrated in Fig. 7D and F, arrows).These data suggest that Hh responding cells in our transgenic mammary model are slow cycling.

In addition, double immunofluorescence staining for integrin β3 and PCNA showed that PCNA positive cells did not coincide with integrin β3 expressing cells in WAP Shh/Ptch1 lacZ basal

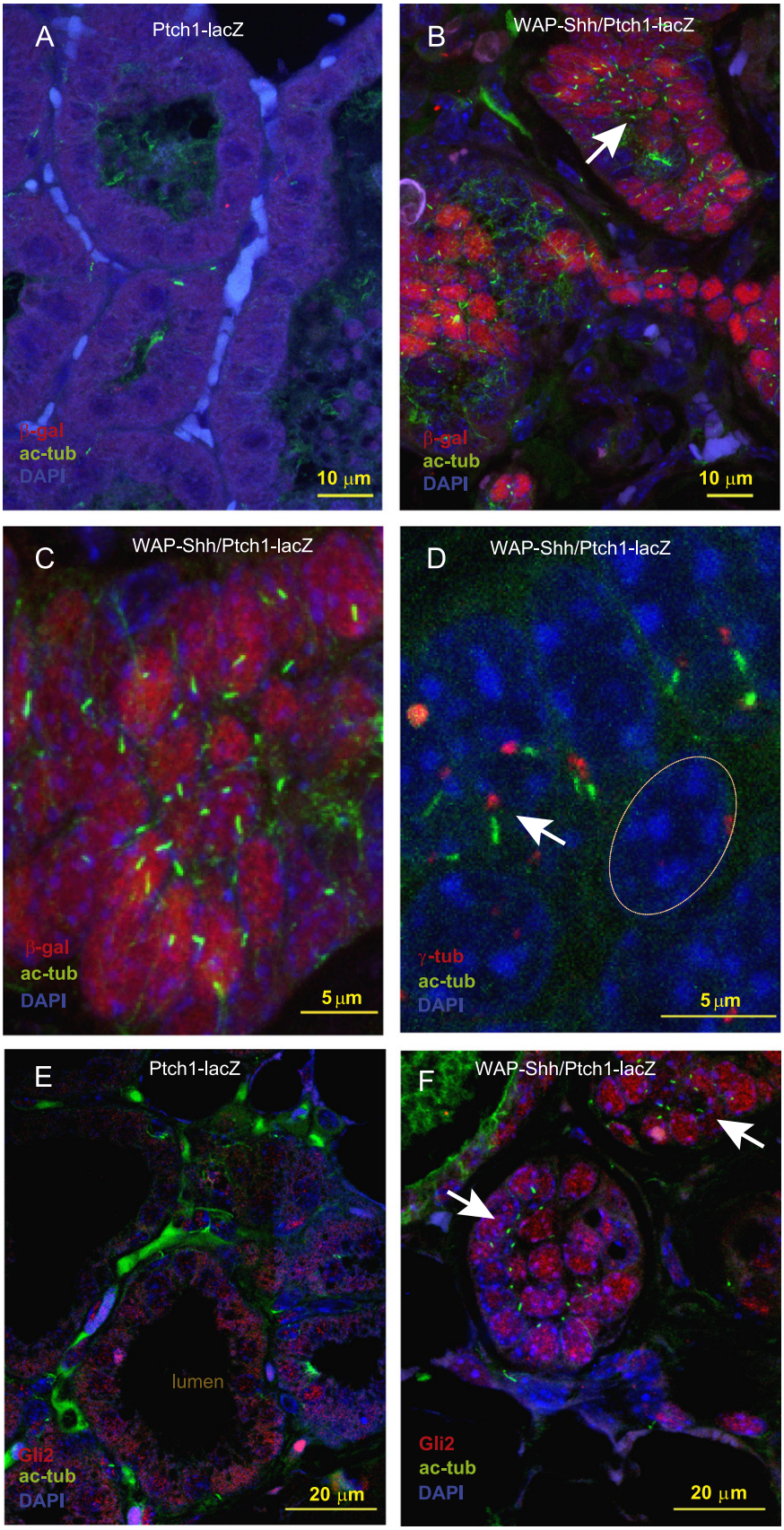

Fig. 8. Hh-responsive cells in WAP-Shh/Ptch1-lacZ mammary tissue present primary cilia.Confocal laser scanning immunofluorescence detection of primary cilia in paraffin sections from Ptch1-lacZ control (A and E) and WAP-Shh/Ptch1-lacZ basal cell hyperplasias (B–D and F). (A–C) β-gal/acetylated tubulin staining: (B) β-gal-expressing cells exhibited primary cilia (white arrow) as detected by acetylated tubulin staining, while β-gal negative cells (blue nuclei) do not present cilia; (C) higher magnification of (B) showing correlation between Hh responsiveness (β-gal expression) and cilia presence; (D) Acetylated tubulin/gamma tubulin immunofluorescence analysis identifying the green shafts as cilia. Gamma-tubulin, a marker for centrioles and basal body of cilia, is localized near the cilia marker acetylated tubulin (arrow). The ellipse delineates a DAPI-stained nucleus. (E, F) Acetylated tubulin/Gli2 in Ptch1-lacZ control (E) and WAP-Shh/Ptch1-lacZ basal cell hyperplasia (F) staining showing that ciliated cells in WAP-Shh/Ptch1-lacZ hyperplasias present nuclear Gli2 staining (F, arrow).

mammary hyperplasias (Fig. 7I, J, arrows). The PCNA (+)/β3 integrin( ) to PCNA (+)/β3 integrin(+) cell ratio was 11.88 ± 0.1 1 (Fig. 7C) suggesting that, within the Hh responding cells, the subset expressing integrin β3 proliferated at a even slower rate.

Hedgehog responsive cells in WAP Shh/Ptch1 lacZ mammary tissue presented primary cilia.

Hh signaling is tightly related to a cellular organelle, the primary cilium, a dynamic structure that grows in some quiescent cells. Double immunostaining for ciliary acetylated tubulin and β gal showed abundant cilia in areas with many Hh responsive cells in WAP Shh/Ptch1 lacZ mammary hyperplasias (Fig. 8B, white arrow, magnified in C). A complete correlation between hedgehog responsiveness (β gal positive cells) and cilia presence was observed. In contrast, very scarce cilia were found in myoe pithelial and stromal cells of β gal negative Ptch1 lacZ lactating control mammary tissue (Fig. 8A). To confirm that these structures are bona fide primary cilia we double stained with ciliary basal body marker gamma tubulin and ciliary shaft marker acetylated tubulin, and found adjacent location of both markers as predicted (Fig. 8D, arrow).

Double immunofluorescence staining for Gli2 and acetylated tubulin showed colocalization of nuclear Gli2 and cilia in WAP Shh/Ptch1 lacZ dysplastic areas (Fig. 8F), further showing that Hh pathway activation correlates with cilia presence in the mammary gland.

Discussion

We have investigated Hh signaling in the lactating mammary gland and determined the effects of Hh pathway misactivation in the mammary tissue. Our model is based on transgenic over expression of Hh ligand in luminal epithelium, the site of Hh expression in mouse and human mammary gland and breast tumors (Gallego et al., 2002; Lewis et al., 2001; O'Toole et al., 2011) and in other organs (Podlasek et al., 1999). We have established our Shh mammary overexpression model in a hemi zygous Ptch1 background, breeding WAP Shh mice to mice carrying a Ptch1 lacZ knock in null allele. This allows visualiza tion of pathway activation through detection of the nuclear β gal protein. The presence of this null allele also results in itself in limited activation of the Hh pathway (Goodrich et al., 1997). In fact, development of mammary ductal dysplasias and hyperplasias in postpubescence virgin mice carrying this allele constituted the first evidence of the implication of the Hh pathway in mammary biology (Lewis et al., 1999). Our model, based on lactation driven expression of Shh, has allowed us to focus on the Hh pathway activity in lactating mammary glands.

Hh responding cells accumulated in areas that resembled benign human mammary intraductal proliferative lesions (ductal hyperplasia or lobular intraepithelial hyperplasia) found in human mammary pathologies (Cardiff and Wellings, 1999). These lesions were found after extensive induction of Shh transgene expression in WAP Shh/Ptch1 lacZ transgenic mam mary glands, consistent with the Hh pathway being overtly active in the mammary tissue only when constitutive repression is over ridden by overexpression or mutation of pathway elements. This is in agreement with previous observations that suggest that Hh unresponsiveness is characteristic of mammary tissues (Hatsell and Frost, 2007). Evidence of functional activation of the Hh pathway in the dysplastic areas was provided by the nuclear localization of Gli2, the initial executor of Hh induced transcriptional activation, in ciliated cells from these areas that also overexpressed p63, a marker for Hh responsiveness in our model.

We have found that secretory mammary epithelium derived Shh protein does not activate the Hh pathway in the cells that produce Shh in an autocrine fashion. Instead, Hh responding cells were located adjacent to secretory epithelial cells in a distinct differentiation compartment, suggesting that Hh signaling in mammary tissue, in the conditions established in our model, is mostly intraepithelial and paracrine. Paracrine Hh signaling has been described during the development of the digestive tract and prostate (Mao et al., 2010; Yu et al., 2009) and during ovarian, colon (Yauch et al., 2008), prostate (Shaw et al., 2009), pancreas (Bailey et al., 2009) and breast (O'Toole et al., 2011) cancer progression. In all mentioned cases, Hh secreted by epithelial cells activates the Hh pathway in adjacent mesenchymal cells. However, intraepithelial Hh signaling has also been described and might play a role in stimulating the proliferation of progenitor cells of organs and tumors (Beachy et al., 2004; Landsman et al., 2011; Watkins et al., 2003).

The signaling mechanism that we describe here involves two different types of epithelial mammary cells: a mature secretory luminal cell that expresses Shh and a basally located Hh responding cell found in hyperplastic ducts and alveoli. Although we found that the stroma is thicker and collagen I deposition is increased in areas adjacent to the hyperplastic Hh responsive areas, we did not detect β gal staining in mesenchymal cells in these regions, suggesting that these extracellular matrix altera tions are secondary to the activation of the pathway in adjacent epithelia. According to these data, intraepithelial Hh signaling, rather than signaling involving epithelial mesenchymal interac tions, seems to be prevalent in the mammary tissue. A recent report shows that the Hh pathway is activated in mesenchymal cells in the stroma of breast tumors in response to tumor derived Hh ligands (O'Toole et al., 2011). This apparent discrepancy with our data might be explained by a different behavior of the tumoral stroma, a highly dynamic environment that changes with tumor progression, and the stroma of the non neoplasic mammary gland (Mukherjee et al., 2006). In spite of the presence of basal cell hyperplasias and dysplasias in their mammary glands, multiparous WAP Shh/Ptch1 lacZ female mice did not develop spontaneous mammary tumors, hindering the study of Hh signaling in mouse mammary gland tumorigenesis. Aging transgenic and non transgenic littermates developed occasional squamous metaplasias, but Hedgehog pathway activity was not found in these lesions, as determined by the absence of β gal immunostaining (data not shown).

Analysis of the expression pattern of differentiation markers suggests that Hh responding cells might be related to progenitor cells. We found a strong expression of basal markers p63, and keratins 5, 14 and 17 in Ptch1 lacZ expressing cells, remarkably higher than that found in adjacent mammary myoepithelial cells. This upregulation of basal markers contrasts with the absence of smooth muscle actin expression, readily detectable in the adjacent myoepithelium. The expression of basal keratins in the mammary progenitor pool has been previously described (Dontu et al., 2003; Shackleton et al., 2006; Stingl et al., 2006) and overexpression of K5 in combination with absence of SMA has been documented as a hallmark of the putative progenitor cell (Bocker et al., 2002; Gudjonsson et al., 2005; Kittrell et al., 2011). Despite their basal location, the cuboidal morphology of Hh responding cells clearly differentiated them from the flattened, polarized mature myoepithelial cells. Overexpression and sym metrical deposition of collagen IV in these cells, reflecting their loss of baso lateral polarity, further illustrates their incomplete differentiation.

The expression of luminal progenitor cell markers integrin β3 and keratin 15 (Asselin Labat et al., 2007; Celis et al., 2007) in partially overlapping groups of Hh responding cells, as well as the sporadic expression of progenitor cell marker K6 (Bu et al., 2011) in adjacent cells, suggests that Hh signaling misactivation might cause the expansion of different populations of mammary pro genitor cells. This expansion could reflect an increased prolifera tion rate of preexistent progenitor cells. A role for Hh signaling in stem and progenitor cell proliferation has been previously described (Li et al., 2008; Liu et al., 2006). Increased presence of cells expressing basal markers (but not SMA) in the second and subsequent lactations may be the result of impaired differentia tion of lobular progenitor cells or parity identified mammary epithelial cells (PI MECs) (Wagner et al., 2002; Boulanger et al., 2005) to functional myoepithelial cells in developing lobules. Altered differentiation of mammary progenitor cells has been reported in a recently described transgenic model of ligand independent activation of the Hh pathway in mammary epithe lium (Visbal et al., 2011). Other mechanisms might also contri bute to progenitor cell accumulation. Dedifferentiation of myoepithelial cells to an undifferentiated basal progenitor phe notype and subsequent slow proliferation of these dedifferen tiated cells might occur, resulting in the substitution of the mature myoepithelium by a single layer of cuboidal basal cells, as we observe in hyperplastic ducts. Hh signaling has been associated with dedifferentiation of mature β cells to undiffer entiated pancreatic tumoral cells (Landsman et al., 2011). Similar mechanisms might operate in other organs such as prostate, pancreas, lung and liver, in which regulation of the progenitor cell compartment by intraepithelial hedgehog signaling has been reported (Karhadkar et al., 2004; Lau and Hebrok, 2010; Sicklick et al., 2006; Watkins et al., 2003).

Another characteristic of the Hh responding cells in the mammary epithelium is the presence of cilia. This organelle has recently emerged as a crucial regulator of signal transduction (Goetz et al., 2009). Genetic data proved that it is necessary for Hh pathway activation (Huangfu et al., 2003) and Hedgehog respon siveness correlates with the presence of primary cilia in prostatic and pancreatic normal and tumoral tissue (Zhang et al., 2009; Cervantes et al., 2010). In the mature mammary gland, cilia have been found in epithelial and mesenchymal cells (McDermott et al., 2010) and mutant mice lacking cilia present mammary branching defects that correlate with a significant decrease in Gli1 expression (McDermott et al., 2010).

We showed herein for the first time the correlation between Hh responsiveness and the presence of cilia in the mammary gland, since Hh responding cells were ciliated and cilia were very rare in non responding areas. Mesenchymal cells in the extra cellular matrix adjacent to Hh responsive epithelia did not dis play a significant increase of primary cilia, consistent with the absence of β gal staining in these cells.

Cilia dependent regulation of other signaling pathways has also been reported. In particular, modulation of Wnt signaling by sequestration of Wnt pathway components to the cilia has recently been shown (Ajima and Hamada, 2011) and Wnt activa tion occurred in the mammary glands of mutant mice lacking cilia (McDermott et al., 2010). Consistently, the Wnt pathway was not active in the ciliated, Hh responsive mammary epithelial cells of our transgenic model, as determined by the location of β catenin, a Wnt signal transduction effector was absent from the nucleus and cytoplasm of these cells. This suggests that Wnt pathway activation, a common event in mouse mammary hyperplasias, does not underlie the morphological alterations found in Hh responsive ciliated regions.

Recent data established that ciliogenesis and cell cycle pro gression are mutually exclusive, since recruitment of a centriole to the cilium basal body is not compatible with mitotic spindle formation (Pan and Snell, 2007) and ciliogenesis might play a role in cell cycle control (Kim et al., 2011). Consistently, we found that Hh responding cells were slow cycling, as assessed by PCNA expression. This observation is in agreement with a previous report showing that epithelial proliferation in a transgenic mouse model expressing a constitutively active Smoothened (MMTV Smo M2) in mammary epithelium, was restricted to Smo M2 negative mammary epithelial cells adjacent to Smo M2 expressing cells (Visbal et al., 2011).

Using a transgenic model that allows to clearly distinguish Hh ligand expressing and Hh responding cells we have found that Hh pathway activation causes increased progenitor cell markers expression and altered differentiation in the mammary gland via a mechanism involving the interaction between two different types of mammary epithelial cells. This intraepithelial paracrine interaction might be at work in pathological mammary situations such as mammary benign hyperplasias, that constitute a risk factor for subsequent tumor malignant pathologies (Dupont and Page, 1985), and in the self renewal of the cancer stem cells during tumor progression.

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Appendix A. Supplementary materials

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